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Survival in Soils of an Herbicide-Resistant *Pseudomonas putida* Strain Bearing a Recombinant TOL Plasmid

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***Pseudomonas putida* EEZ15(pWW0-EB62) is a phosphinothricin (PPT)-resistant strain with a recombinant TOL plasmid which allows the strain to grow on *p*-ethylbenzoate. The survival of this strain in sterile agricultural soils depends on the physicochemical properties of the soil. The recombinant pWW0-EB62 plasmid and its catabolic functions were stable for periods of up to 1 month in bacteria introduced in unamended soils and only conferred selective advantage to the host bacteria without the plasmid or with the natural pWW0 plasmid when the soils were amended with low amounts of *p*-ethylbenzoate. The addition to soils of aromatics that are cometabolized by *P. putida* EEZ15(pWW0-EB62) had a detrimental effect on the survival of the bacteria, whereas low amounts of aromatics that are not metabolized by this bacterium had no effect on their survival. Survival of *P. putida* EEZ15(pWW0-EB62) was better at 4 and 25°C than at 37°C. The host bacterium carrying the recombinant pWW0-EB62 plasmid was established in unsterile soils.**

The genetic manipulation of aromatic catabolic pathways in bacteria is a powerful approach for improving the rate of degradation of recalcitrant xenobiotic chemicals (24). This approach has resulted in the construction of recombinant bacteria capable of degrading a wide variety of alkyltoluenes and alkylbenzoates (1, 25), chlorobenzoates (23, 27, 28), and chlorosalicylates (16). However, little work has been done to test the survival of these genetically engineered microorganisms under nonoptimal conditions, i.e., conditions other than those used in laboratory cultures (3, 7, 10, 20, 32). The potential use of bacteria with chromosomally encoded or with natural or chimeric plasmid-encoded catabolic pathways to augment or initiate in situ biodegradation depends on the stability of the genetic information harbored and the successful survival of the organisms under specific conditions. Furthermore, such information is crucial in providing safety assurances relative to the environmental risk of such applications.

Natural conditions may present a number of problems for laboratory-designed bacteria reintroduced into the environment. Low substrate concentrations may not support the growth of these specialized strains, or the introduced organisms may abandon metabolism of the contaminant in favor of other substrates (11). Naturally occurring toxins, predators, and interspecific competition for nutrients may all reduce the survival of the introduced strain (14, 17). On the other hand, alternate carbon sources available in natural ecosystems may enhance the survival of introduced strains and hence the degradation of contaminants (30).

The tracking of bacteria under laboratory culture conditions and in soil and aquatic microcosms is facilitated by the use of markers, including antibiotic ones, which have been widely used for laboratory strains. Other markers such as resistance to herbicides and pesticides, etc., may be desirable to facilitate tracking of bacteria which may be released into the environment. Phosphinothricin (PTT) is a potent herbicide that inhibits glutamine synthetase from many different sources (5, 8, 15). The inhibition of this enzyme leads to glutamine depletion and rapidly results in the prevention of cell growth, leading thereafter to death of the microorganism (15). In this study, we report the survival in different soils of a PTT-resistant *Pseudomonas putida* strain bearing the recombinant TOL plasmid pWW0-EB62.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *P. putida* 2440 (*hsdR*) was described in an earlier publication (9). *P. putida* EEZ15 is a PTT-resistant derivative of *P. putida* 2440, which is described in Results. *P. putida* PaW340 (Nal^r Sm^r Trp) was provided by S. Harayama (University of Geneva, Geneva, Switzerland). The plasmids used in this study were pWW0 (*p*-xylene$^+$ *p*-toluate$^+$ *p*-ethyltoluene$^-$ *p*-ethylbenzoate$^-$) (33) and pWW0-EB62 (*p*-xylene$^+$ *p*-toluate$^+$ *p*-ethyltoluene$^+$ *p*-ethylbenzoate$^+$) (1). The latter plasmid carries two mutations at the *xylS* gene and the *xylE* gene. The *xylS4* allele encodes for a mutant XylS regulator that activates transcription from the TOL *meta* cleavage pathway operon with *p*-ethylbenzoate. The *xylE6* allele encodes for a catechol 2,3-dioxygenase that is resistant to inactivation by its substrate *p*-ethylcatechol (1).

Bacteria were grown routinely on LB medium or on M9 minimal medium (19) with 5 mM $ClNH_4$ or 10 mM KNO_3 as a nitrogen source and with one of the following carbon sources: glucose (0.5%, wt/vol), acetate, benzoate or alkylbenzoates (5 mM), or *m*-xylene vapors.

Soil experiments. The agricultural soils used (Table 1) were placed in jars that contained 70 g of soil. Before use, the soils were sifted through a 4-mm-mesh metal sieve. Unless otherwise indicated, soils were sterilized in an autoclave under a vapor stream (120°C for 1 h) three times; the mass was allowed to cool completely between each step (6). All experiments were performed at a moisture content of about 10 to 15%. One milliliter of cells in 50 mM phosphate buffer was added to the jars containing sterile soils to a density of about 10^8 CFU/g of soil unless otherwise indicated. Soils were tested in duplicate. To estimate culturable cells (CFU), 10 g of soil was added to 90 ml of 50 mM phosphate buffer and shaken at 30°C for 1 h. This was the initial dilution, and

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TABLE 1. Some chemical properties of the soils used in this study

Soil no.	Type	Organic matter[a]	C/N	$CaCO_3$[a]	pH	Crop
1	Cambisol	0.63	10.2	23.4	7.5	Olives
2	Fluvisol	1.72	14.1	6.2	7.6	Potatoes
3	Regosol	0.47	9.7	32.2	8.3	Wheat
4	Regosol	0.64	9.5	41.7	8.3	Wheat
5	Solonchak[b]	0.84	11.6	23.4	7.9	
6	Luvisol	0.48	9.3	26.7	7.8	Grapes
7	Cambisol	0.46	7.7	12.0	8.0	Olives
8	Regosol	2.71	9.7	30.8	7.5	Olives
9	Fluvisol	2.53	9.5	8.4	8.5	Maize
10	Fluvisol	2.25	10.1	6.4	8.0	Custard apples

[a] Values are given as percentages of weight.
[b] This soil contained 28.1% as $CaSO_4$. Values are from Pérez-Pujalte and Prieto-Fernandez (21).

while still being shaken, it was used to obtain a range of dilutions so that we could detect between 10^1 and 10^8 CFU/g of soil. In each CFU determination, at least three different dilutions were spread in duplicate on selective plates. The values given in Results represent the average number of readily countable CFU from all dilutions and from duplicate soil jars. Standard deviations were in the range of 5 to 30% of the given values. Selective plates for *P. putida* EEZ15(pWW0-EB62) contained 5 mM *p*-ethylbenzoate and 1 mM PTT.

Preparation of cell extracts and determination of enzyme activities. Cell extracts of *P. putida* 2440 and *P. putida* EEZ15 were prepared as follows: 100 ml of cells grown on M9 minimal medium was harvested by centrifugation (5,000 × *g* for 5 min), washed once in 50 mM phosphate buffer (pH 7.0), and sonicated at 4°C for 4 min, with bursts of 30 s and intervals of 30 s between bursts. After centrifugation (12,000 × *g* for 5 min) to remove the cell debris, the supernatant was used as the cell extract. PTT *N*-acetyltransferase activity was estimated spectrophotometrically by measuring the rate of formation of the free coenzyme A sulfhydryl group coincident with the transfer of the acetyl group to PTT, as described by Bartsch and Tebbe (2). For glutamine synthetase determinations, cells were grown on nitrate as a nitrogen source. Glutamine synthetase activity was measured by the transferase assay in cell extracts (31) or in permeabilized whole cells (23). Units are given as micromoles of product per minute per milligram of protein.

RESULTS

Isolation and characterization of PTT-resistant *P. putida* 2440 bacteria. To estimate the degree of resistance of *P. putida* 2440 to the herbicide PTT, cultures of the bacteria on minimal medium with glucose as a carbon source and increasing amounts of PTT (0.1 to 2.2 mM) were set up. We found that concentrations of 1 mM PTT fully inhibited growth of the bacteria. This was ultimately the result of the loss of glutamine synthetase in the cells exposed to the herbicide. The higher the concentration of PTT, the faster glutamine lost synthetase activity. The addition of 1 mM PTT to cultures of *P. putida* 2440 led to the loss of about 50% glutamine synthetase in 3 min, activity becoming negligible after 15 min (Fig. 1). To select mutants of *P. putida* 2440 resistant to PTT, cells were spread on minimal medium plates with glucose as a carbon source and with 1 mM PTT; the mutation rate was found to be about 6×10^{-7} per cell per generation. One randomly selected clone was chosen for further characterization and called *P. putida* EEZ15. The mutation in EEZ15 is highly stable, as 100% of the cells conserved the phenotype after growing in the absence of selective pressure for longer than 50 generations. *P. putida* EEZ15 grows on minimal medium with glucose as a carbon source and 1 mM PTT with a generation time of 90 min, which is similar to the growth rate in the absence of PTT and is also equivalent to that of the parental strain in the absence of PTT with the same carbon source.

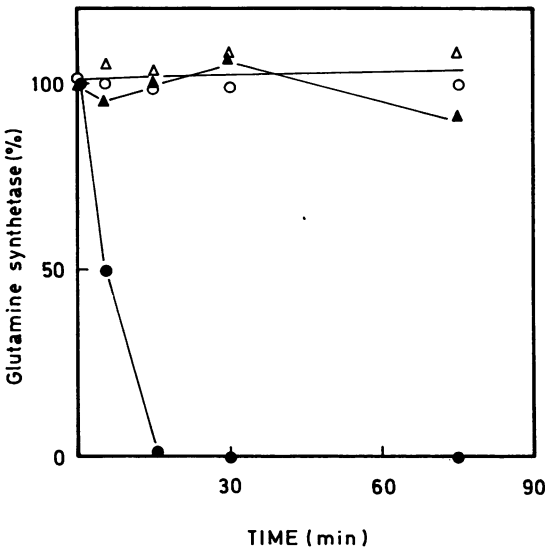

FIG. 1. Glutamine synthetase levels in vivo in *P. putida* 2440 and *P. putida* EEZ15 in the presence and in the absence of 1 mM PTT. *P. putida* 2440 (○, ●) and *P. putida* EEZ15 (△, ▲) were grown on minimal medium with glucose as a carbon source and nitrate as a nitrogen source. At $t = 0$, the culture was split in two halves; one was supplemented with 1 mM PTT (●, ▲) and the other was kept as a control (○, △). At the indicated times, cells were permeabilized (23) and glutamine synthetase activity was determined (31). Glutamine synthetase activity of 100% corresponds to 540 ± 20 mU mg of protein^{-1}.

P. putida EEZ15 growing on glucose in the presence of 1 mM PTT conserved high levels of glutamine synthetase activity for longer than 75 min (Fig. 1). However, in vitro assays revealed that the glutamine synthetases of the mutant and wild-type strains were equally sensitive to PTT, suggesting that the mutation was not in the glutamine synthetase itself (Fig. 2). PTT *N*-acetyltransferase and PTT-oxidase activities were negligible in the wild-type and the mutant strains. Therefore, these enzymes cannot account for the resistance of EEZ15 to the herbicide. The resistance of *P. putida* EEZ15 to PTT seems to be due to blockage of the entry of the herbicide into the cells (unpublished data).

Transference of plasmid pWW0-EB62 from the auxotrophic *P. putida* PaW340 to *P. putida* EEZ15 was then carried out. *P. putida* EEZ15(pWW0-EB62) was selected on minimal medium with *p*-ethylbenzoate as a carbon source and 1 mM PTT. As expected, this bacterium grew on minimal medium with *m*-xylene, *m*-toluate, or *p*-ethylbenzoate as a carbon source, in the presence and in the absence of PTT.

Survival of *P. putida* EEZ15 bearing the plasmid pWW0-EB62 in different soils. The survival of *P. putida* EEZ15(pWW0-EB62) was first assayed in 11 different ster-

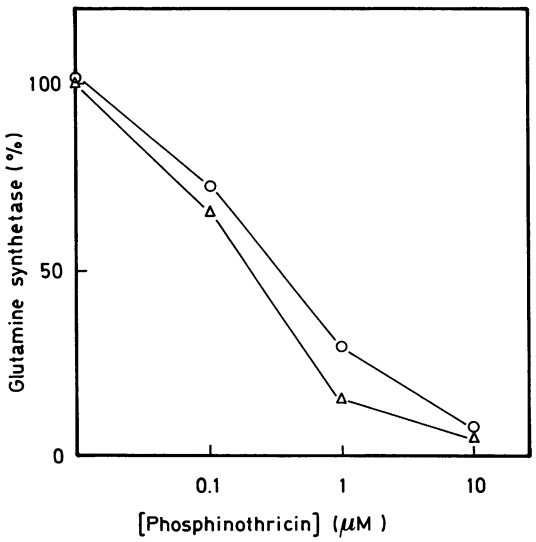

FIG. 2. In vitro effect of PTT on the glutamine synthetase activity of *P. putida* 2440 and *P. putida* EEZ15. Cell extracts of *P. putida* 2440 (○) and *P. putida* EEZ15 (△) were prepared as described in Materials and Methods. Glutamine synthetase activity was determined in the presence of increasing concentrations of PTT. Glutamine synthetase activity of 100% corresponds to 600 mU mg of protein^{-1}.

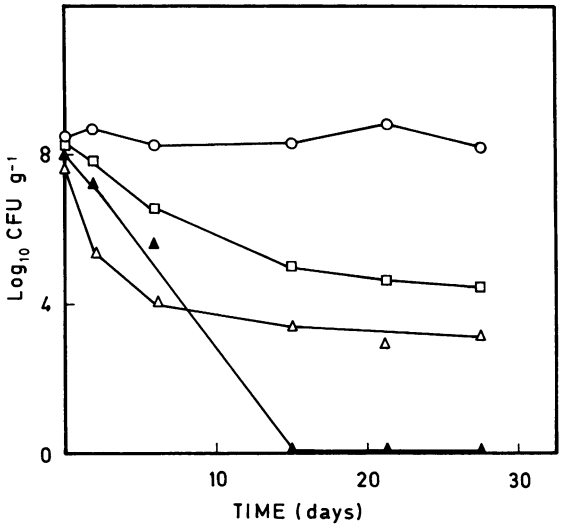

FIG. 3. Survival of *P. putida* EEZ15(pWW0-EB62) in the laboratory in different soils. *P. putida* EEZ15(pWW0-EB62) was introduced in soils from the Granada area (cambisol soil-1 [□], regosol soil-3 [△], and fluvisol soil-10 [○]) and in a soil contaminated with heavy metals (▲) to a density of about 10^8 CFU/g of soil. The numbers of CFU were determined at the time of inoculation and thereafter on minimal medium plates with *p*-ethylbenzoate as a carbon source and 1 mM PTT.

ilized soils (Tables 1 and 2). *P. putida* EEZ15(pWW0-EB62) was introduced into the sterile soils to a cell density of about 10^8 CFU/g of soil. Thereafter, the number of culturable bacteria (CFU) was estimated by plating out bacteria on minimal medium with *p*-ethylbenzoate as a carbon source and with 1 mM PTT. We found that in the fluvisol soil-10, the number of CFU remained practically constant in experiments lasting 1 month (Fig. 3). In all of the other soils from the Granada area, the number of bacteria decreased during the first 2 weeks; thereafter, the number of CFU remained relatively constant. In all cases, the number of CFU stabilized at 10^3 to 10^6 CFU/g of soil. Figure 3 shows the survival of the strain in a typical Mediterranean soil (cambisol-1) and in the regosol soil-3. In a soil contaminated with heavy metals (grams per kilogram of soil: Zn, 16.5; Pb, 2.6; and Cu, 6.7; from a zinc desert field area in Lommel, Belgium), the number of CFU decreased steadily with time until it fell below our detection limits (Fig. 3).

When *m*-xylene or *m*-toluate was used instead of *p*-ethylbenzoate as the selective carbon source, the numbers of CFU were similar to those determined in *p*-ethylbenzoate in experiments run for a month. Moreover, 100% of the colonies that were found on plates with *p*-ethylbenzoate grew on plates with *m*-toluate or *m*-xylene as a carbon source, and vice versa. This suggests that the information for the pWW0-EB62 plasmid catabolic pathways is highly stable in unamended soils. In addition, loss of the recombinant plasmid pWW0-EB62 in *P. putida* EEZ15 maintained in soils for a month seemed not to occur, as the numbers of CFU determined on glucose plus PTT (for which a chromosomally encoded catabolic route is required) and *p*-ethylbenzoate plus PTT were also similar. Of the colonies found on glucose plates, 100% grew on plates with *p*-ethylbenzoate as the sole carbon source.

TABLE 2. Granulometric analysis of some of the soils used in this study

Soil no.	Type	% Soil that was:		
		Sand	Silt	Clay
1	Cambisol	57.42	27.61	14.97
3	Regosol	60.18	36.70	3.12
10	Fluvisol	61.08	32.47	6.45

To further confirm that the *P. putida* EEZ15(pWW0-EB62) recovered from soils conserved the properties of the strain introduced in the soils, the growth rates of the recovered cells were determined on minimal medium with *p*-ethylbenzoate, benzoate, and glucose. The generation times estimated as 75, 60, and 90 min on *p*-ethylbenzoate, benzoate, and glucose, respectively, were similar to those determined under comparable conditions for the strain that had not been introduced in soils. *P. putida* EEZ15(pWW0-EB62) recovered from soils were also reintroduced in the same or different soils, and their survival was followed again in a month-long experiment. The survival of this bacteria was similar to that described above for bacteria introduced in soil for the first time (data not shown).

For cambisol soil-1 and regosol soil-3, we tested whether the presence of the recombinant plasmid pWW0-EB62 conferred advantages to the host cells over cells bearing either no plasmid or the wild-type pWW0 plasmid. In soils without additions, the survivals of *P. putida* EEZ15(pWW0-EB62), *P. putida* EEZ15(pWW0), and *P. putida* EEZ15 were similar; however, the addition of 0.1% (wt/wt) *p*-ethylbenzoate favored survival of the strain bearing pWW0-EB62, while it had detrimental effects on the survival of the two other strains. Figure 4 shows the results obtained in regosol soil-3, which were similar to those obtained in cambisol soil-1 (data not shown).

Multiplication of *P. putida* EEZ15(pWW0-EB62) in soils. To determine whether *P. putida* EEZ15(pWW0-EB62) multi-

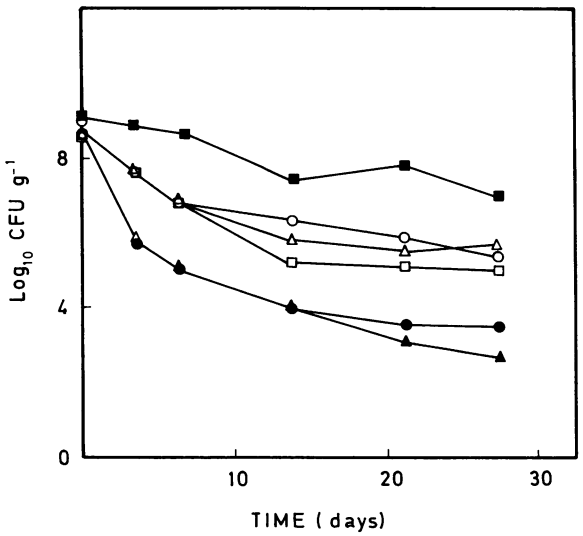

FIG. 4. Survival of *P. putida* EEZ15 bearing and not bearing wild-type or recombinant plasmid pWW0-EB62 in regosol soil-3. Independent jars containing 70 g of unamended regosol soil-3 (○, △, □) or the same soil amended with 0.01% (wt/wt) *p*-ethylbenzoate (●, ▲, ■) were inoculated with *P. putida* EEZ15 (△, ▲), *P. putida* bearing wild-type pWW0 plasmid (○, ●), and *P. putida* bearing pWW0-EB62 plasmid (□, ■). *P. putida* EEZ15, *P. putida* EEZ15(pWW0), and *P. putida* EEZ15(pWW0-EB62) were counted on minimal medium containing 1 mM PTT plus benzoate, *m*-methylbenzoate, or p-ethylbenzoate, respectively, as a carbon source.

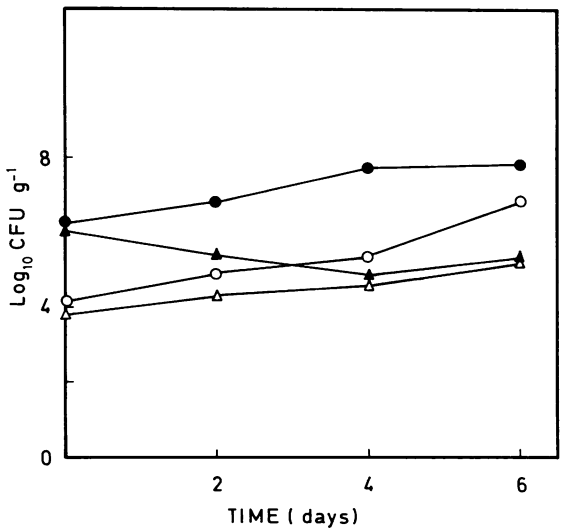

FIG. 5. Multiplication of *P. putida* EEZ15(pWW0-EB62) in fluvisol soil-10 and cambisol soil-1. Unamended fluvisol soil-10 (○, ●) and cambisol soil-1 (△, ▲) samples were inoculated with *P. putida* EEZ15(pWW0-EB62) to densities of about 10^4 and 10^6 CFU/g of soil. The numbers of CFU were determined at the time of inoculation and thereafter as indicated.

plies in soil, duplicate jars containing cambisol soil-1 and fluvisol soil-10 were loaded with bacteria grown on minimal medium with *p*-ethylbenzoate to a cell density of about 10^4 and 10^6 CFU/g of soil. We observed that, in cambisol soil-1, the number of bacteria tended to stabilize at 10^5/g of soil (8×10^4 to 2×10^5), i.e., we observed a slight increase when bacteria were introduced at the lower density and a slight decrease when bacteria were introduced at the higher cell density (Fig. 5).

In fluvisol soil-10, the number of bacteria increased in both cases, tending to stabilize at 10^7 CFU/g of soil (7×10^6 to 2.5×10^7) (Fig. 5). This suggests that the manipulated microorganism bearing the recombinant plasmid is able to multiply in soils until it reaches a plateau which probably represents the carrying capacity of the soil for the bacteria.

Survival of *P. putida* EEZ15(pWW0-EB62) in soils amended with aromatic carboxylic acids. Three different aromatic carboxylic acids, *p*-ethylbenzoate, *p*-chlorobenzoate, and salicylate, were added to soils in which *P. putida* EEZ15(pWW0-EB62) had been introduced to a density of about 10^8 CFU/g of soil. These aromatics were chosen because all three are effectors for the XylS4 protein (25) encoded by the mutant gene *xylS4* borne by the recombinant pWW0-EB62 plasmid (1), and therefore all compounds can induce the synthesis of the pWW0-EB62 plasmid *meta* cleavage pathway. However, this bacterium cannot metabolize salicylate because it lacks the necessary enzymatic machinery. *p*-Chlorobenzoate is metabolized only to 2-chloromuconic acid semialdehyde by *P. putida* EEZ15(pWW0-EB62); in this biotransformation, the bacterium does not obtain energy and the resulting intermediate is toxic for the cell (26, 27). In contrast, *p*-ethylbenzoate can be mineralized by *P. putida* bearing pWW0-EB62.

Cambisol soil-1, regosol soil-3, and fluvisol soil-10, unamended or amended with *p*-ethylbenzoate (0.01, 0.1, and 1 g/100 g of soil [wt/wt]) were inoculated with about 10^8 CFU of *P. putida* EEZ15(pWW0-EB62) per g of soil. In cambisol soil-1 and regosol soil-3 the results were similar, and Fig. 6 shows the results in cambisol soil-1. Low concentrations of *p*-ethylbenzoate (0.01 or 0.1% [wt/wt]) favored survival of the bacterium, while higher concentrations (1%) had a det-

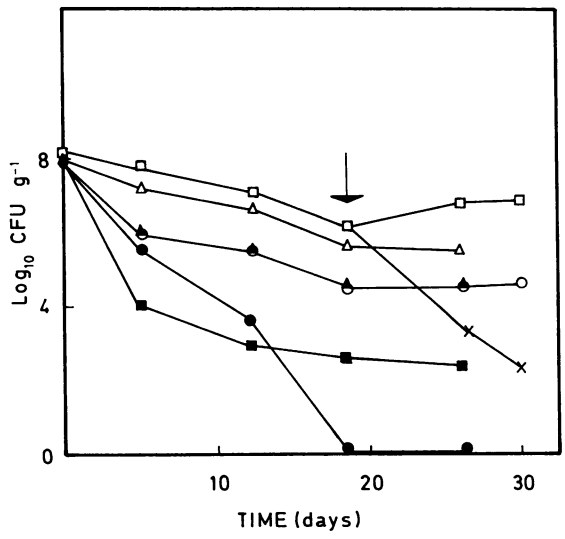

FIG. 6. Survival of *P. putida* EEZ15(pWW0-EB62) in cambisol soil-1. *P. putida* EEZ15 at a density of about 10^8 CFU/g of soil was introduced in jars containing cambisol soil-1 unamended (○) or amended with either 0.01% (wt/wt) (△), 0.1% (wt/wt) (□), or 1% (wt/wt) (●) *p*-ethylbenzoate, with 0.1% salicylate (▲), or with 0.1% *p*-chlorobenzoate (■). The arrow indicates that at day 18 half of the soil in the jar (□) was transferred to another jar, to which *p*-ethylbenzoate was added to a concentration of 1% (wt/wt) *p*-ethylbenzoate (×).

TABLE 3. Survival of *P. putida* EEZ15(pWW0-EB62) in a typical Mediterranean cambisol soil-1 and fluvisol soil-10 at different temperatures[a]

p-Ethylbenzoate	Survival of *P. putida* in:					
	Cambisol soil-1			Fluvisol soil-10		
	4°C	25°C	37°C	4°C	25°C	37°C
−	10^{-4}	5×10^{-5}	10^{-6}	5×10^{-1}	10^{-1}	3×10^{-3}
+	10^{-3}	10^{-4}	10^{-8}	1	1	10^{-3}

[a] Two independent duplicate jars were inoculated with *P. putida* EEZ15(pWW0-EB62) at a density of 2×10^8 to 5×10^8 CFU/g of soil. The values given are averages of the ratios between the number of CFU after 30 days of incubation at the temperature indicated and the number of CFU determined at the time of inoculation. Standard deviations were in the range of 5 to 20% of the given values.

rimental effect on survival. In fact, after 18 days, the number of bacteria fell below detectable limits. This detrimental effect was also observed when 1% *p*-ethylbenzoate was added to soils in which *P. putida* EEZ15(pWW0-EB62) had been exposed for 18 days to lower levels of *p*-ethylbenzoate (Fig. 6).

In fluvisol soil-10, the low concentrations of *p*-ethylbenzoate had no significant effect on survival, while 1% of *p*-ethylbenzoate clearly reduced the survival of the bacterium (data not shown).

In cambisol soil-1, salicylate and *p*-chlorobenzoate were introduced at a concentration of 0.1% (wt/wt). Salicylate had no apparent effect on the survival of *P. putida* EEZ15 (pWW0-EB62), while *p*-chlorobenzoate had a detrimental effect. This was especially evident in the first 2 weeks, although the number of CFU stabilized thereafter (Fig. 6).

Effect of temperature on survival of EEZ15(pWW0-EB62) in soils. *P. putida* EEZ15(pWW0-EB62) was introduced into cambisol soil-1 and fluvisol soil-10, both either amended or unamended with *p*-ethylbenzoate (0.1% [wt/wt]), and incubated at either 4, 25, or 37°C. In the typical Mediterranean cambisol soil-1, survival was better at 4 and 25°C than at 37°C. The presence of *p*-ethylbenzoate had some beneficial effect on survival at 4 and 25°C. Incubating *P. putida* EEZ15(pWW0-EB62) at 37°C markedly reduced cell survival even in the presence of *p*-ethylbenzoate (Table 3). In fluvisol soil-10, survival at 4 and 25°C was also better than at 37°C. However, survival in fluvisol soil-10 at the highest temperature was on the order of 10^{-3}, while in cambisol soil-1 survival was on the order of 10^{-6} to 10^{-8}.

Survival of *P. putida* EEZ15(pWW0-EB62) in nonsterile soils. The presence of indigenous microorganisms in cambisol soil-1, regosol soil-3, and fluvisol soil-10 was estimated at about 10^7 to 10^9 microorganisms per g of soil when the number of CFU was estimated on LB medium plates and on minimal medium plates with glucose as a carbon source and ammonium as a nitrogen source. The number of microorganisms able to use *p*-ethylbenzoate as the sole source of carbon and energy per gram of soil was less than 10^4. No microorganisms able to grow on *p*-ethylbenzoate and resistant to 1 mM PTT were found in these soils. To test survival of *P. putida* EEZ15(pWW0-EB62) in these soils, this bacterium was inoculated at a density of about 10^8 CFU per g of soil in soils supplemented and unsupplemented with 0.1% *p*-ethylbenzoate. Survival of the bacterium bearing the recombinant plasmid pWW0-EB62 was followed by counting CFU on minimal medium with *p*-ethylbenzoate and 1 mM PTT. In fluvisol soil-10, the CFU of the bacteria remained relatively

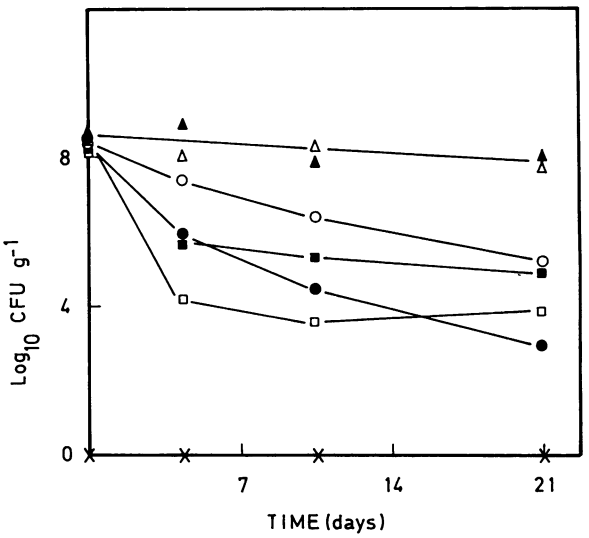

FIG. 7. Survival of *P. putida* EEZ15 in unsterile soils amended or unamended with *p*-ethylbenzoate. About 10^8 CFU of *P. putida* EEZ15 was added to unsterile cambisol soil-1 (○, ●), regosol soil-3 (□, ■), and fluvisol soil-10 (△, ▲) either unamended (○, □, △) or amended (●, ■, ▲) with 0.1% (wt/wt) *p*-ethylbenzoate. The numbers of CFU were determined on minimal medium plates with 5 mM *p*-ethylbenzoate and 1 mM PTT at the time of inoculation and thereafter.

constant over time regardless of the presence of *p*-ethylbenzoate (Fig. 7). In contrast, in cambisol soil-1 and regosol soil-3 the number of CFU decreased with time regardless of the addition of *p*-ethylbenzoate to the soils. It is worth noting that the decay in CFU in cambisol soil-1 was faster in the presence of *p*-ethylbenzoate than in its absence (Fig. 7). This contrasts with observations in sterile soils. However, in regosol soil-3, the presence of low concentrations of *p*-ethylbenzoate favored the survival of the manipulated bacteria, as was also the case in sterile soils (Fig. 7).

DISCUSSION

The efficacy of genetically engineered microorganisms in the in situ removal of chemicals from the environment depends upon their survival and performance in natural ecosystems. Once a microorganism able to degrade a recalcitrant chemical is constructed in the laboratory, it must be subjected to a series of experiments to evaluate its survival and performance in different ecosystems. In addition, it may be useful to evaluate the factors that enhance or inhibit its performance in the environment.

To facilitate the tracking of a *P. putida* bacteria bearing a recombinant TOL plasmid, we first isolated mutants of the host bacteria resistant to the herbicide PTT. PTT was chosen because it is a potent inhibitor of glutamine synthetase (5, 8, 15), and although bacteria resistant to PTT have been isolated from soils by others (2), in the soil samples we worked with no microorganisms resistant to PTT and able to use *p*-ethylbenzoate as the sole source of carbon and energy were found. Therefore, we consider PTT resistance as a useful marker for tracking the host *P. putida* bacteria bearing a recombinant TOL plasmid which allows these microorganisms to grow on *p*-ethylbenzoate. The characteristic of resistance to PTT in *Pseudomonas* spp. is stable in soils and under laboratory culture conditions. The mutation in *Pseu-*

domonas bacteria prevents the in vivo inactivation of glutamine synthetase by PTT, although in vitro, glutamine synthetase from the wild type and the mutant were equally sensitive to herbicide inhibition. The resistance to PTT in *P. putida* EEZ15 is probably due to prevention of the entry of the herbicide into the cell (25a).

The survival of *P. putida* EEZ15 without plasmid, or bearing either the natural plasmid pWW0 or the recombinant plasmid pWW0-EB62, was similar in unamended soils. However, when soils were amended with low amounts of *p*-ethylbenzoate, the bacteria bearing the recombinant plasmid survived better; therefore, the strain bearing pWW0-EB62 enjoyed an obvious advantage in *p*-ethylbenzoate-amended soils. The beneficial effect of *p*-ethylbenzoate is due to the mineralization of these compounds in soils by the bacterium bearing pWW0-EB62 (7a). We found no direct correlation between the survival of the strain and soil type, organic content, C/N ratio, or calcium content of the soil. It seems likely that several parameters that define the physicochemical characteristics of a soil determine bacterial survival, and hence the survival of a given strain will vary depending on the soil.

Although pWW0-EB62 conferred a selective advantage to bacteria in soils amended with *p*-ethylbenzoate, it should be noted that above certain levels this compound is toxic to the bacteria and markedly reduces its survival. Thus, bacteria able to thrive in highly polluted environments would be potential hosts for manipulated catabolic pathways.

We also observed that the presence of TOL plasmid (whether manipulated or not) may have deleterious effects on survival when bacteria are confronted with compounds that may be transformed into dead-end products. This has been observed when the bacteria are exposed to *p*-chlorobenzoate, which is transformed into its corresponding chloromuconic acid semialdehyde (26, 27). As chemical pollution is often complex, involving more than a single chemical, this needs to be considered when designing manipulated bacteria, which should be as versatile as possible. The construction of methyl- and haloaromatic degraders is nevertheless feasible, as shown by Rojo et al. (28).

The stability of the plasmid pWW0-EB62 in *P. putida* EEZ15 introduced in soils was confirmed by the following facts: (i) similar numbers of CFU were estimated on media with different carbon sources, whose assimilation requires either chromosomally encoded routes or plasmid-encoded pathways, and (ii) *P. putida* EEZ15(pWW0-EB62) recovered from soils on glucose plates conserved the ability to grow on *p*-ethylbenzoate. In addition, the catabolic functions of the plasmid pWW0-EB62 in *P. putida* EEZ15 introduced in soils remained stable for periods of up to a month, since (i) the numbers of CFU estimated by using an upper pathway substrate (e.g., *m*-xylene) and a *meta* pathway substrate (e.g., *p*-ethylbenzoate, *m*-toluate) were similar, and (ii) bacteria selected on *p*-ethylbenzoate were also able to grow on *m*-xylene and vice versa.

It is worth noting that, although the wild-type pWW0 and recombinant pWW0-EB62 plasmids are highly stable in the laboratory even after the bacteria have been grown on rich medium for many generations, loss of the plasmid occurs in bacteria growing on benzoate, which is assimilated through a chromosomally encoded *ortho* cleavage pathway by *P. putida* (12, 13). We have observed that *P. putida* EEZ15 (pWW0-EB62) multiplies well in unamended soils, and that under these conditions the recombinant plasmid is maintained in the bacteria. Therefore, although the copy number of the TOL plasmid is low, loss of the plasmid is prevented thanks to its efficient partition.

Multiplication of *Pseudomonas aureofaciens* marked with the *Escherichia coli lacZY* tracking systems has been shown to occur in plant rhizospheres. The engineered strain was as efficient as the nonengineered one in establishing high population levels (7). Although the number of examples is not great, it seems that the multiplication of genetically engineered microorganisms in soils occurs in a fashion similar to that in the parental strain (7, 29; Fig. 5).

Soil temperature is another important factor which affects the persistence of bacteria in the natural environment. *P. putida* EEZ15(pWW0-EB62) survives better at low temperatures than at higher temperatures. The amendment of soils with *p*-ethylbenzoate benefited survival at 4 and 25°C but not at 37°C. This may be because the XylS protein is not synthesized at 37°C (22), and therefore synthesis of the catabolic enzymes does not occur at this temperature. Thus, temperature-dependent synthesis of enzymes and regulators and their thermostability should also be considered when designing a new bacteria for mineralization purposes.

Our preliminary results suggest that *P. putida* EEZ15 (pWW0-EB62) can be established in unsterile soils both in the absence and in the presence of selective pressure. This has also been observed with *ice* mutant *Pseudomonas syringae* strains (18) and in rhizobia (4). The effect of the introduction of bacteria on the indigenous population and biotic factors that may affect the persistence of the bacteria (parasitism and predation and production of bacteriocins and other microbial antagonists, etc.) are other important factors that require further evaluation.

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